Allergy 2004: 59: 772-780
Printed in UK. All rights reserved



ALLERGY

## Original article

# A bispecific antibody against human IgE and human FcγRII that inhibits antigen-induced histamine release by human mast cells and basophils

**Background:** FcγRIIB are low-affinity immunoglobulin (Ig)G receptors that we previously demonstrated to negatively regulate IgE-induced mast cell activation when coaggregated with FcεRI. Here, we engineered and characterized a bispecific reagent capable of coaggregating FcγRIIB with FcεRI on human mast cells and basophils.
**Methods:** A bispecific antibody was constructed by chemically crosslinking one Fab' fragment against human IgE and one Fab' fragment against human FcγRII. This molecule was used to coaggregate FcεRI with FcγRII on human mast cells and basophils sensitized with human IgE antibodies, and the effect of coaggregation was examined on mediator release upon challenge with specific antigen.
**Results:** When used under these conditions, this bispecific antibody not only failed to trigger the release of histamine by IgE-sensitized cells, but it also prevented specific antigen from triggering histamine release. Comparable inhibitions were observed with mast cells and basophils derived *in vitro* from cord blood cells and with peripheral blood basophils.
**Conclusions:** The bispecific antibody described here is the prototype of similar molecules that could be used in new therapeutic approaches of allergic diseases based on the coaggregation of activating receptors, such as FcεRI, with inhibitory receptors, such as FcγRIIB, that are constitutively expressed by mast cells and basophils.

**S. W. Tam[1], S. Demissie[1], D. Thomas[1], M. Daëron[2]**
[1]Tanox Inc., Houston, TX, USA; [2]Laboratoire d'Immunologie Cellulaire et Clinique, Institut Biomédical des Cordeliers, Paris, France

Key words: basophils; FcγRIIB; FcεRI; mast cells; negative regulation; therapeutic antibody.

Sun W. Tam
Charles River Laboratories
57 Union Street
Worcester, MA 01608
USA

Accepted for publication 23 June 2003

Although many other cells participate in the complex network of inflammatory processes that lead to the development of allergies, mast cells and basophils play a critical role in the initiation of immunoglobulin (Ig)E-dependent allergic reactions. When activated by IgE and allergen, mast cells and basophils secrete inflammatory mediators and cytokines that act on vascular and muscular cells and recruit inflammatory cells. These, in turn, secrete inflammatory mediators and recruit other inflammatory cells, and the process goes on. Following an IgE-induced, mast cell-dependent immediate-type reaction, waves of inflammatory cells thus become sequentially attracted and activated that altogether generate a long-lasting inflammation. As a consequence, new means of controlling mast cell activation would provide a novel therapeutic approach of allergic diseases by preventing the initiation of the inflammatory response. It happens that mast cells and basophils are constitutively equipped with regulatory receptors that could be used in therapeutics, provided that appropriate ligands be engineered.

The activation of mast cells is positively regulated by several membrane receptors, the prototype of which is the high-affinity IgE receptor FcεRI (1). These receptors possess one or several intracytoplasmic molecular motifs named Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) (2). When these receptors are aggregated at the cell surface by multivalent ligands (e.g. a multivalent allergen that binds to receptor-bound IgE antibodies in the case of FcεRI), their ITAMs are phosphorylated by protein tyrosine kinases of the src family (3). Phosphorylated ITAMs provide docking sites for a variety of SH2 domain-containing cytosolic molecules (4) which can interact with each others in a spatially and temporally organized fashion. These molecules include enzymes, substrates and adapters which, altogether, build up a dynamic signaling complex under aggregated receptors (5). Intracellular signals generated in this complex trigger an array of metabolic pathways that lead to an increase in the cytosolic $Ca^{2+}$ concentration and the activation of transcription factors (6). Ultimately, the exocytotic machinery is activated, leading to the release of granular preformed mediators, and cytokine genes are transcribed, leading to the synthesis and secretion of their products.

The activation of mast cells is negatively regulated by several membrane receptors the prototypes of which are the low-affinity IgG receptors FcγRIIB (7). These receptors possess one or several intracytoplasmic molecular motifs named Immunoreceptor Tyrosine-based Inhibition Motifs (ITIMs) (8, 9). When these receptors are coaggregated with ITAM-bearing receptors (e.g. by an allergen-IgG antibody complex that binds simultaneously to FcεRI-bound IgE antibodies via the allergen and to FcγRIIB via the Fc portion of IgG antibodies (10), their ITIMs are phosphorylated by the same src protein tyrosine kinases that phosphorylate ITAMs (11). Phosphorylated ITIMs also provide docking sites for SH2 domain-containing cytosolic molecules. The specificity of phosphorylated ITIMs, however, is different from that of phosphorylated ITAMs: they recruit selectively phosphatases. These are either SH2 domain-containing phosphatidylinositol 5-phosphatases (SHIPs), in the case of FcγRIIB (12–15), or SH2 domain-containing protein tyrosine phosphatases (SHPs), in the case of other inhibitory receptors (16, 17). When recruited in the signaling complex, these phosphatases interfere with activation signals. As a consequence, the $Ca^{2+}$ response is impaired (18, 19) and signals that lead to the activation of transcription factors are extinguished (20). Ultimately, both the release of preformed mediators and the secretion of cytokines are inhibited (10). The validity of negative regulation of mast cell activation by FcγRIIB demonstrated *in vitro* was confirmed *in vivo* by using genetically modified mice. Indeed, FcγRIIB-deficient mice exhibited enhanced IgG- and IgE-induced local and systemic anaphylactic reactions (21).

Although well established in mice, FcγRIIB-dependent negative regulation of mast cell activation is poorly documented in humans. One reason is that there are several types of FcγRII in humans, which are usually coexpressed on the same cells (7). These are the inhibitory FcγRIIB, as in mice, and the activating FcγRIIA and/or C that are unique to humans (22). FcγRIIA/C are capable of activating mast cells, as shown when transfected and aggregated in the rat mast cell model RBL-2H3 (8). They indeed possess an ITAM in their intracytoplasmic domain (2), instead of an ITIM. Another difficulty is that no monoclonal antibody (mAb) specific for human FcγRIIB is available. As a consequence, which type(s) of FcγRII are expressed by human mast cells and basophils, and in which proportions, is unclear. In a previous work, we showed that human FcγRIIB had the same inhibitory properties on IgE-induced mast cell activation as murine FcγRIIB, when transfected in RBL-2H3 cells (8). In addition, we showed that FcγRII constitutively expressed by human blood basophils also inhibited IgE-induced histamine release by these cells (8). Finally, we showed that a bispecific molecule capable of coaggregating mouse IgE with mouse FcγRIIB profoundly inhibited IgE-induced secretory responses in RBL-2H3 transfectants (10). Based on these previous results, we constructed a bispecific molecule capable of coaggregating human IgE with human FcγRII. We report here that this molecule inhibited antigen-induced secretory responses of human mast cells and basophils sensitized with human IgE antibodies. Our findings provide the grounds of a new immunotherapeutic approach for allergic diseases.

## Methods

### Cells

*Cultured human cord blood-derived mast cells.* Human cord blood-derived mast cells (CBMCs) were derived from human cord blood $CD34^+$ cells grown in the presence of stem cell factor, interleukin (IL)-6, and IL-10 (PeproTech, Rocky Hill, NJ), as modified from the procedure of Saito et al. (23) and described in Cho et al. (24). Briefly, heparin-treated umbilical cord blood was purchased from Advanced Bio-technologies (Columbia, MD). The cord blood sample was diluted 1 : 4 in phosphate-buffered saline (PBS) supplemented with 2% bovine serum albumin (Sigma-Aldrich, St Louis, MO) and 0.6% citrate, loaded onto Ficoll-Histopaque (Sigma-Aldrich), and centrifuged twice at 200 *g*. The interface containing mononuclear cells was mixed with Dynabeads M-450 CD34 according to manufacturer's protocol (Dynal, Lake Success, NY). The $CD34^+$ cells were magnetically separated and later detached from the beads. The washed mononuclear cells were resuspended at $1–5 \times 10^5$ cells/ml in culture medium consisting of Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies/GIBCO/BRL, Rockville, MD), 20% fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine, 50 mM 2-ME, 100 U/ml penicillin, 100 mg/ml streptomycin and 10 mg/ml gentamicin (GIBCO/BRL), supplemented with 80 ng/ml stem cell factor, 50 ng/ml IL-6, and 5 ng/ml IL-10. The cytokine-supplemented medium was replaced on a weekly basis. Cells were harvested after 8–9 weeks of culture. Over 94% of these cells were mast cells as assessed by staining with a mAb directed against mast cell tryptase (Chemicon International, Temecula, CA).

*Cultured human cord blood-derived basophils.* Human $CD34^+$ cord blood cells were purchased from Poietics Inc. (BioWhittaker, Walkersville, MD). The thawed mononuclear cells were resuspended $1–5 \times 10^5$ cells/ml in culture medium supplemented with 1 ng/ml recombinant human IL-3 and 10 ng/ml recombinant human TGF-β1 (PeproTech Inc.). The cytokine-supplemented medium was replaced on a weekly basis. Cells were harvested after 4 weeks of culture.

*Human basophil-containing peripheral blood mononuclear cells.* Peripheral blood lymphocytes were purified from heparinized human blood buffy coat from healthy donors as described (25). The heparinized blood was mixed 1 : 4 part of RPMI media (Gibco) and centrifuged at 200 *g* for 10 min at 10°C to remove the red cells. The supernatant was mixed 1 : 4 part of histopaque (Sigma-Aldrich) and the mixture was centrifuged at 500 *g* for 45 min at 10°C to separate the lymphocyte layer. Cells were washed twice at 200 *g* for 10 min at 10°C in RPMI, and cell viability was assessed by trypan blue staining. About 1–2% of peripheral blood mononuclear cells (PBMCs) were basophils.

*Daudi cells.* The human Burkitt lymphoma cell line, Daudi, was maintained in RPMI 1640 (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), 2 mM L-glutamine, 50 mM 2-mercaptoethanol (2-ME), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO/BRL).

### Antibodies

The mouse anti-human FcγRII AT10 mAb (26) was purified from ascitic fluid, harvested from (BALB/c × CBA) F1 mice injected i.p. with the AT10 hybridoma cells, using ammonium sulfate precipitation and ion-exchange chromatography. The mouse anti-human IgE mAb E10.10.3, generated at Tanox, was purified from culture supernatant from hybridoma cells. Purified monoclonal mouse IgE (IgE-3) was purchased from BD PharMingen (San Diego, CA). The human IgE anti-gp120 mAb SE44 was generated in a B cell transfectoma, containing hybrid epsilon and kappa genomic DNA of human Cε region and κ chains and the H and L chain V regions of the murine mAb BAT123, which reacts with the gp120 envelope protein of HIV-1 (27). Goat anti-mouse (GAM) $F(ab')_2$ was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

### Bispecific antibody

AT10 $F(ab')_2$ fragments were prepared by pepsin (Sigma-Aldrich) digestion of AT10 IgG antibodies at pH 4.1 according to Lamoyi and Nisonoff (28). E10.10.3 $F(ab')_2$ fragments were prepared from E10.10.3 IgG antibodies by pepsin digestion in 20 mM sodium acetate pH 4.5 for 48 h at 37°C using immobilized pepsin beads (Pierce Inc., Rockford, IL). A bispecific molecule containing Fab' fragments of the two mouse mAbs was prepared by linking half-cysteine residues via thioether bonds using the bifunctional cross-linking agent, o-phenylenedimaleimide (o-PDM) (Sigma-Aldrich) as described (26, 29). The $F(ab')_2$ fragments from the two antibodies diluted at 10 mg/ml in 0.2 M Tris-HCl buffer, pH 8.0, containing 10 mM ethylenediaminetetraacetic acid (EDTA) were reduced by the addition of 20 mM 2-ME for 30 min at 30°C. The samples were chilled to 4°C and reduced F(ab') SH were separated from the reducing agent by gel filtration through a G25 column in 50 mM sodium acetate buffer, pH 5.3, containing 0.5 mM EDTA. A half volume of 12 mM o-PDM dissolved in chilled dimethylformamide was then added to one of the two mouse $F(ab')_{SH}$ preparations. After 30 min, the maleimidated $F(ab')_{mal}$ were separated from other solutes by passage through Sephadex G-25 column. They were then added immediately to the other $F(ab')_{SH}$ preparation in a molar ratio of 1 : 1.3 of $F(ab')_{mal}$ : $F(ab')_{SH}$ and concentrated to 5 mg/ml by ultrafiltration under nitrogen using a Diaflo membrane in a chilled Amicon chamber (Amicon, Beverly, MA). After incubation for 18 h, the pH of the reaction mixture was adjusted to 8.0 using 1 M Tris-HCl, before reducing with 20 mM 2-ME for 30 min at 30°C and alkylating with 25 mM iodoacetamide. Finally, the bispecific antibody was separated from other products by passage through Ultrogel AcA44 (IBF Biotechnics, Paris, France) equilibrated in 0.2 M Tris-HCl, 10 mM EDTA, pH 8.0.

### High-pressure liquid chromatography analysis

The bispecific antibody was checked for purity by rapid size fractionation in a high-pressure liquid chromatography (HPLC) system (Amersham Pharmacia, Piscataway, NJ). The fractionation was performed at 0.5 ml/min in a Superdex 200 column (Amersham Pharmacia) equilibrated in 0.2 M phosphate, pH 7.0, containing 10 mM sodium azide. Each assessment requires approximately 30 min, monitored with a 280 nm interference filter (Amersham Pharmacia). The profile of the bispecific antibody was then compared with a molecular weight standard (Amersham Pharmacia) run to approximate the protein size.

### Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) plates (Immulon 2; VWR, So. Plainfield, NJ) were coated by an overnight incubation at 4°C with 1 μg/ml human IgE SE44. Plates were saturated with Blotto (5 g dry milk, 0.05% Tween 20 in PBS buffer) for 1 h at room temperature. The wells were then washed four times in 0.05% Tween 20 diluted in PBS. Serial dilutions of AT10, E10.10.3 $F(ab')_2$, or bispecific antibody were added to wells in duplicates and plates were incubated at room temperature for 2 h. Plates were washed in 0.05% Tween 20, and horseradish peroxidase-conjugated GAM kappa antibodies (Jackson ImmunoResarch) were added for 1 h at room temperature. Plates were washed again in 0.05% Tween 20, and the presence of enzyme-conjugated antibodies was revealed by adding 3,3', 5,5'-tetramethylbenzidene (TMB) substrate (BD PharMingen) for 15 min at room temperature. The reaction was stopped with 2 M $H_2SO_4$, and the optical density of the reaction mixture was measured using an ELISA spectrophotometer at 450 nm.

### Indirect immunofluorescence

Daudi cells were incubated for 30 min at 0°C with 25 μg/ml bispecific antibody, AT10 $F(ab')_2$, or $F(ab')_2$ fragments of an irrelevant isotype control antibody (26). The cells were washed and incubated for 30 min at 0°C with 0.3 mg/ml FITC-labeled polyclonal rabbit anti-mouse Ig antibodies (BD PharMingen). After a final wash, fluorescent cells were analyzed in the presence of 1 mM $NaN_3$ (to prevent internalization) using a fluorescence-activated cell sorter (FACS III; Becton Dickinson, Mountain View, CA).

### Histamine release

*Cells sensitized with murine IgE.* Aliquots of $1 \times 10^5$ human CBMCs were resuspended in a 1 : 1 mixture of RPMI medium: histamine release ionic buffer (Immunotech/Beckman Coulter, Fullerton, CA). They were incubated for 1 h at 37°C with 10 μg/ml mouse IgE SE44 and with or without various concentrations of AT.10. Cells were washed twice and challenged for 1 h at 37°C with 10 μg/ml prewarmed (15 min at 37°C) GAM $F(ab')_2$.

*Cells sensitized with human IgE.* Aliquots of $1 \times 10^5$ 9-week-old CBMCs, of $5 \times 10^4$ 4-week-old cord blood-derived basophils (CBBs) or of $1 \times 10^5$ PBMCs were incubated for 1 h at 37°C with 10 μg/ml human IgE anti-gp120 (mAb SE44), washed and incubated for 30 min at 37°C with or without indicated concentrations of bispecific antibody, 10 μg/ml E10.10.3 or 10 μg/ml AT10. Cells were washed again and challenged for 1 h at 37°C with 100 ng/ml gp120-conjugated ovalbumin (27).

*Histamine measurements.* At the end of the reaction, supernatants were collected by centrifugation and acylated according to the Immunotech protocol. Histamine was measured in supernatants using the Immunotech human histamine competitive ELISA kit.

## Results

### FcγRII inhibit IgE-induced histamine release when coaggregated with FcεRI on human CBMCs

In preliminary immunofluorescence studies, we found that, in addition to high-affinity IgE receptors (FcεRI),

human CBMCs express low-affinity IgG receptors. Indeed, $F(ab')_2$ fragments of the mouse mAb AT10 (26), which recognize both FcγRIIA/C and FcγRIIB (8), bound to CBMCs (our unpublished results). $F(ab')_2$ fragments of the FcγRIIA/C-specific mouse mAb IV.3 (8) also bound to CBMCs. These observations indicated that human CBMCs express the cell-activating receptors FcγRIIA/C. They neither indicated nor excluded that these cells express the inhibitory receptors FcγRIIB. As a consequence, one could not predict whether aggregating FcγRII on human CBMCs would trigger cell activation, or not; and whether coaggregating FcεRI with FcγRII would result in a potentiation, or an inhibition of IgE-induced secretory responses.

To answer this question, CBMCs were sensitized with mouse IgE or without, and incubated with AT10 or without. After being washed, they were challenged with GAM Ig $F(ab')_2$ fragments, and histamine released in the supernatant was measured by ELISA. Mouse IgE alone, AT10 alone or the combination of both induced no histamine release over background. FcεRI aggregation, induced by GAM $F(ab')_2$ in CBMCs sensitized with IgE, triggered histamine release, whereas FcγRII aggregation, induced by GAM $F(ab')_2$ in CBMCs incubated with AT10, did not. The coaggregation of FcεRI with FcγRII, induced by GAM $F(ab')_2$ in CBMCs sensitized with IgE and incubated with AT10, triggered no histamine release either (Fig. 1).

That histamine release was induced neither upon FcγRII aggregation nor upon coaggregation of FcγRII with FcεRI suggests that human CBMCs do express FcγRIIB and that FcγRIIB could inhibit both FcγRIIA/C-dependent activation [induced by AT10 + GAM $F(ab')_2$], and FcγRIIA/C + FcεRI-dependent activation [induced by IgE + AT10 + GAM $F(ab')_2$]. The same was previously observed using the same experimental protocol on human peripheral blood basophils (8). Human cultured mast cells and blood basophils could therefore be used to assess the potential inhibitory properties of a molecule capable of coaggregating FcεRI with FcγRII.

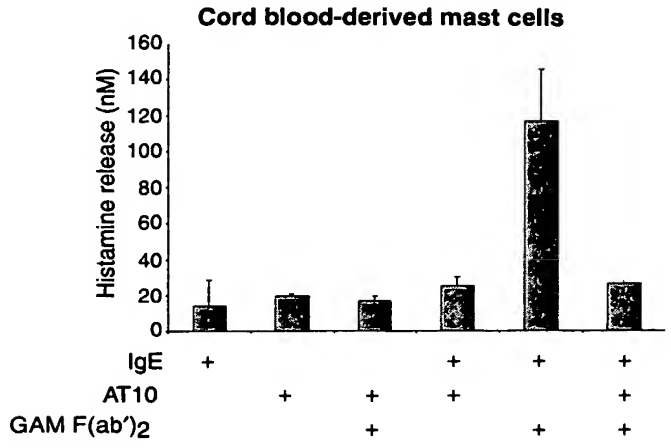


*Figure 1.* Inhibition of histamine release upon coaggregation of FcεRI with FcγRII on human cord blood-derived mast cells (CBMCs). Aliquots of $1 \times 10^5$ human CBMCs were incubated for 1 h at 37°C with mouse immunoglobulin E and/or mouse mAb anti-human FcγRII antibody AT10. Cells were washed and challenged or not challenged with goat anti-mouse (GAM) $F(ab')_2$. All reagents were used at 10 μg/ml, final concentration. Histamine released into the supernatant was measured by enzyme-linked immunosorbent assay. Histograms show the concentration of histamine (mean ± SE from three independent experiments) in cell supernatants following treatments indicated below the graph.

### Construction of a monovalent bispecific anti-FcγRII/anti-IgE antibody

In order to construct a reagent capable of coaggregating FcεRI with FcγRII on human mast cells and basophils, Fab' fragments were prepared from the mouse mAb anti-human IgE E10.10.3 and from the mouse mAb anti-human FcγRII AT10. The resulting two Fab' fragments were chemically linked to each other using (o-PDM) as described (26, 29). Under the experimental conditions used, only heterodimers could be generated. The hybrid molecule was purified by gel chromatography and characterized by HPLC, by ELISA and by indirect immunofluorescence.

When analyzed by HPLC, this molecule eluted as a predominant peak with a m.w. of about 100 kDa (Fig. 2A). When analyzed by ELISA, it bound onto human IgE-coated plates almost as efficiently as $F(ab')_2$ fragments of the parental mAb E10.10.3. Under the same conditions, AT10, the other parental mAb, did not bind at all to IgE (Fig. 2B). When analyzed by indirect immunofluorescence on the FcγRII-positive Burkitt lymphoma cell line Daudi, the hybrid molecule bound as efficiently as $F(ab')_2$ fragments of the parental mAb AT10 (Fig. 2C).

A 100-kDa molecule, capable of binding both to human IgE and to human FcγRII was therefore generated. Considering the preparation conditions used and its apparent m.w., this bispecific molecule should be composed of one AT10 Fab' fragment and of one E10.10.3 Fab' fragment. This monovalent bispecific molecule, referred to as (AT10XE10) bsAb, was used to coaggregate FcεRI and FcγRII on human mast cells and basophils sensitized with human IgE.

### (AT10XE10) bsAb prevents IgE-induced histamine release by human mast cells and basophils

The effect of (AT10XE10) bsAb on IgE-induced histamine release was examined on three types of human cells. The CBMCs, obtained by culturing human $CD34^+$ cord blood cells in the presence of SCF, IL-6 and IL-10, CBBs, obtained by culturing the same $CD34^+$ cells in the

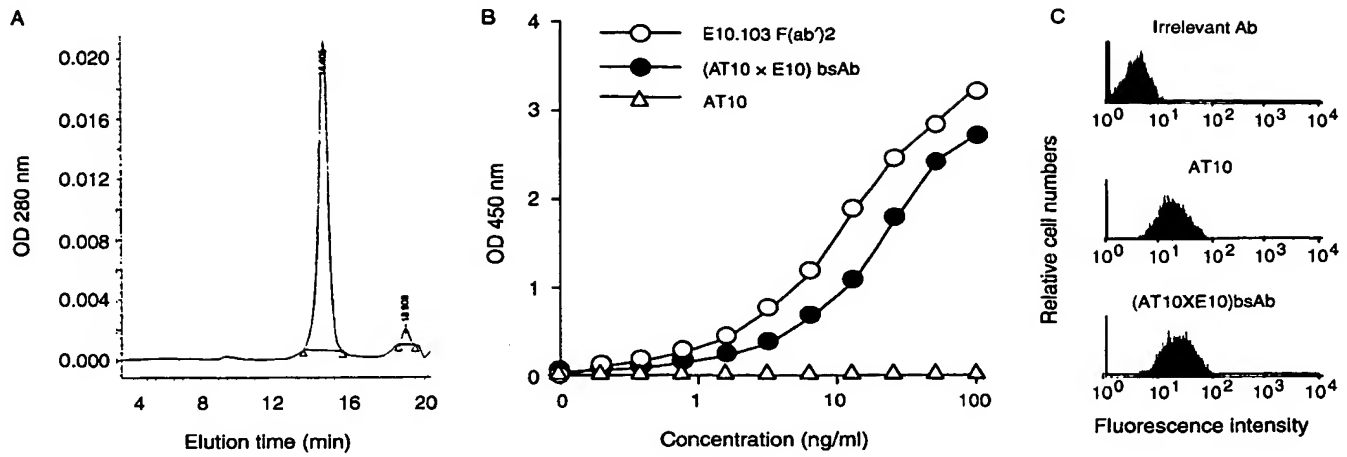


*Figure 2.* Characterization of the [anti-immunoglobulin (Ig)E × anti-FcγRII] bispecific antibody. (A) High-pressure liquid chromatography analysis of (AT10XE10) bsAb. The graph represents the elution profile of (AT10XE10) bsAb, measured by the absorbance at 280 nm, as a function of time. The major peak eluted together with a m.w. marker of about 100 kDa. (B) Enzyme-linked immunosorbent assay analysis of the binding of (AT10XE10) bsAb to human IgE. The binding of serially diluted (AT10XE10) bsAb, E10.10.3 F(ab')2 (anti-IgE) and AT10 (anti-FcγRII) onto human IgE-coated plates was measured by ELISA using horseradish peroxidase-conjugated anti-mouse κ antibodies. The curves represent the absorbance at 250 nm as a function of the dilution of antibodies. (C) Immunofluorescence analysis of the binding of (AT10XE10) bsAb to human Fcγ*RII*. The FcγRII-expressing Burkit lymphoma cells Daudi were incubated with 25 μg/ml (AT10XE10) bsAb, AT10 or an irrelevant isotype control. Cell-bound antibodies were revealed using FITC-rabbit anti-mouse antibodies. Histograms show the fluorescence intensity measured by flow cytometry.

presence of IL-3 and TGF-β, and basophil-containing PBMC, prepared from human buffy coat, were sensitized with human IgE anti-gp120 (clone SE44). After being washed, they were incubated with various concentrations of (AT10XE10) bsAb or with one of the parental antibodies (E10.10.3 or AT10), washed again, and challenged with gp120-conjugated ovalbumin or not. Histamine released into the supernatant was monitored by ELISA.

None of the three cell types released significant amounts of histamine when incubated with (AT10XE10) bsAb and challenged with gp120-ovalbumin or not. As expected, all three cell preparations released histamine when sensitized with IgE anti-gp120 and challenged with gp120-ovalbumin (Fig. 3A–C) or with parental anti-IgE mAb E10.10.3, but not with (AT10XE10) bsAb (Fig. 3A, B). Histamine release was inhibited when cells sensitized with IgE anti-gp120 were incubated

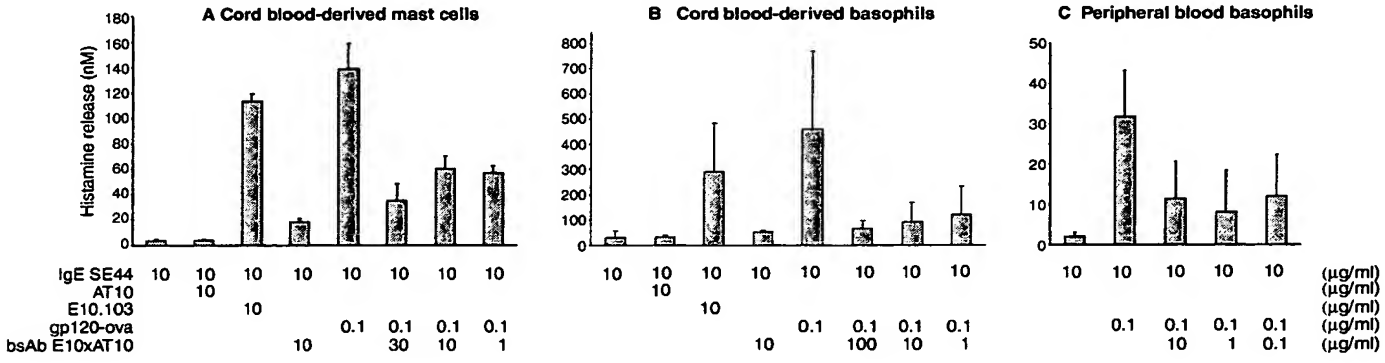


*Figure 3.* Inhibition of histamine release by (AT10XE10) bsAb on human mast cells and basophils. Aliquots of (A) $1 \times 10^5$ cord blood-derived mast cells, (B) $5 \times 10^4$ cord blood-derived basophils and (C) $1 \times 10^5$ basophil-containing peripheral blood mononuclear cells were sensitized with human immunoglobulin (Ig)E anti-gp120 (IgE SE44), washed, incubated or not with indicated concentrations of (AT10XE10) bsAb, washed again, and challenged with medium, parental anti human IgE mAb (E10103) or gp120-conjugated albumin (gp120-ova) as indicated under the graph. Histamine released in the supernatant was measured by enzyme-linked immunosorbent assay. The histograms represent the concentration of histamine (mean ± SE from three independent experiments) in cell supernatants following indicated treatments.

with (AT10XE10) bsAb before challenge with gp120-ovalbumin (Fig. 3A–C). Inhibition was observed in all three cell types, for all concentrations of (AT10XE10) bsAb tested. An incubation of cells with the parental antibody AT10, instead of (AT10XE10) bsAb, induced no significant inhibition (not shown). The coaggregation, by (AT10XE10) bsAb, of FcεRI-bound IgE antibodies with FcγRII on cultured mast cells, on cultured basophils or on peripheral blood basophils, therefore prevented specific antigen from inducing histamine release.

## Discussion

The present paper describes the construction of a bispecific molecule able to coaggregate FcεRI and FcγRII on human mast cells and basophils sensitized with IgE (Fig. 4). This molecule was found to be monovalent and to retain the specificity of both parental antibodies. When used to coaggregate FcεRI-bound human IgE and FcγRII, it prevented antigen from eliciting histamine release. Comparable inhibitions were induced in cultured human mast cells and basophils, as well as in peripheral blood basophils. It follows that, although both ITAM-containing activating FcγRII and ITIM-containing inhibitory FcγRII are expressed on human mast cells and basophils, FcγRIIB-dependent inhibition is dominant and can be exploited, using bispecific antibodies, for therapeutic purposes. Based on these results, similar bispecific antibodies could be tailored aiming at coaggregating FcεRI with other inhibitory receptors expressed on human mast cells and basophils.

FcγRIIB-dependent negative regulation of cell activation is difficult to assess in human mast cells and basophils. First, these cells were not formally demonstrated to express FcγRIIB. Secondly, they express cell-activating FcγRIIA/C, as revealed by the binding of the FcγRIIA/C-specific mAb IV.3 (8). Ideally, one would need antibodies that would specifically recognize FcγRIIB on the cell surface in order to (1) ascertain whether this inhibitory receptor is expressed by mast cells and basophils, and (2) coaggregate

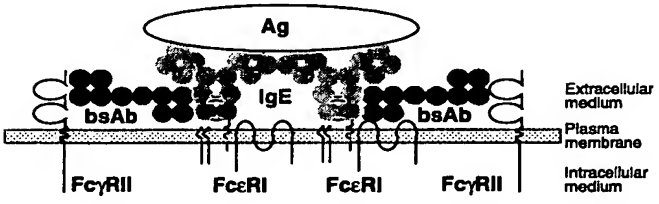


*Figure 4.* Schematic representation of (AT10XE10) bsAb when coaggregating FcεRI with FcγRII on human mast cells or basophils sensitized with human IgE and challenged with specific antigen. FcεRI and FcγRII are coexpressed by human mast cells and basophils. The two receptors are coaggregated on the same cells when (AT10XE10) bsAb binds simultaneously to FcεRI-bound IgE via its E10 Fab' and to FcγRII via its AT10 Fab'.

FcεRI selectively with FcγRIIB. Unfortunately, such antibodies are not available and are unlikely to be generated in view of the highly conserved sequences of the extracellular domains of FcγRIIA, FcγRIIB and FcγRIIC. There is indeed no extracellular amino acid present in FcγRIIB that is not also present in FcγRIIA or FcγRIIC. Under these conditions, one can only coaggregate FcγRII (A/C + B, if any) with FcεRI, hoping that FcγRIIB-dependent inhibition will be dominant over FcγRIIA/C-dependent activation. Supporting this possibility, IgG immune complexes are, in general, unable to activate basophils (30), and indeed, we could not induce histamine release when aggregating FcγRII with the mAb AT10, which binds equally well to FcγRIIA/C and to FcγRIIB (8), on human peripheral basophils or on CBMCs. Moreover, IgE-dependent histamine release was inhibited when FcγRII were coaggregated with FcεRI on the same cells. These data suggest that human CBMCs probably express FcγRIIB, and that FcγRIIB-dependent negative regulation is dominant not only over FcγRIIA/C-dependent cell activation but also over (FcγRIIA/C + FcεRI)-dependent cell activation. Similar results were previously observed in peripheral human basophils (8). One may therefore envision the coaggregation of FcεRI with FcγRII, on cells which express the two receptors, as a possible new therapeutic approach of allergies.

Based on this strategy, a recently published paper reported that a bifunctional fusion protein could inhibit FcεRI-dependent human mast cell and basophil activation (31). The inhibitory properties of this molecule, made of a human IgG1 Fc portion (hinge-$C\gamma_H2$-$C\gamma_H3$) fused to a human IgE Fc portion ($C\varepsilon_H2$-$C\varepsilon_H3$-$C\varepsilon_H4$) via a 15-aminoacid linker, were proposed to result from the coaggregation of FcεRI with FcγRII. The requirement of prolonged incubation periods, the inhibitory effect on syk phosphorylation and the intrinsic inability of the bifunctional molecule to coligate FcγRII with those FcεRI that were sensitized with specific IgE and engaged by antigen made a competitive effect likely to account for inhibition, rather than a coaggregation of FcεRI with FcγRII. In the present work, we constructed a molecule made of one Fab' fragment of an anti-FcγRII antibody (AT10) and one Fab' fragment of an anti-IgE antibody known not to interfere with the binding of IgE to FcεRI (E10.10.3). An anti-human IgE antibody was chosen, instead of an anti-FcεRI antibody, for the following reasons. Allergic patients are expected to have already synthesized allergen-specific IgE antibodies that are bound to FcεRI and, because of the low dissociation rate (32), remained bound to these receptors. More importantly, one anticipates an efficient inhibition of allergen-induced reactions to preferentially target receptors occupied by specific IgE antibodies, rather than unoccupied receptors, as we previously demonstrated that inhibition requires that FcγRIIB be coligated with FcεRI that are aggregated by antigen (10) and thereby provide the protein tyrosine kinase which phosphorylates FcγRIIB

(11). One possible problem resulting from this strategy was that an anti-IgE molecule might activate mast cells sensitized with IgE (both specific and nonspecific). This was not the case since cultured mast cells, cultured basophils or blood basophils did not release significant amounts of histamine when exposed to (AT10XE10) bsAb, whether sensitized or not with human IgE. This molecule is therefore most likely monovalent for both IgE and FcγRII. Indeed, as judged by its apparent m.w., it only contained two Fab' fragments and, because of the preparation conditions used (one partner only was maleimidated), only two Fab' with different specificities could be chemically linked. (AT10XE10) bsAb is bispecific, as demonstrated by its ability to bind to human IgE, as assessed by ELISA, and by its ability to bind to the FcγRII-expressing human Burkitt lymphoma cells Daudi, as assessed by indirect immunofluorescence. Being both monovalent and bispecific, it was used to coaggregate FcεRI with FcγRII on IgE-sensitized human mast cells and basophils and, as a result, it significantly inhibited antigen-induced histamine release. Inhibition indeed similarly affected all three cell types used, i.e. CBMCs, CBBs and peripheral blood basophils, and as little as 1 μg/ml bsAb could inhibit histamine release induced by 10-fold higher concentrations of IgE.

As a consequence of these observations, we would like to propose that bispecific molecules such as the (AT10XE10) bsAb described herein could be humanized and possibly used as therapeutic tools. As demonstrated here, such molecules would be expected to prevent IgE-induced mast cell activation, which determines the onset of allergic diseases. One should point out that, because of the difficulties discussed above, direct evidence that human mast cells and basophils do express FcγRIIB remains to be obtained. It should confirm our functional data. Not all mast cell populations may possibly express FcγRIIB, and in any case with the same FcγRIIB/FcγRIIA ratio. If so, FcγRII-dependent negative regulation may not affect identically these cells, as the ratio of activating and inhibitory receptors can be anticipated to be critical. Interestingly, because of their bispecificity, they would target not only mast cells and basophils, but also monocytes/macrophages (33, 34) and eosinophils (35, 36) which also express both FcγRII and FcεRI, and which are thought to be major effectors in diseases such as asthma and atopic dermatitis. Following the same rationale, we also propose that other bispecific molecules be constructed, that would coaggregate FcεRI with other inhibitory receptors. Three such molecules, gp49B1/HM18 (37), PIR-B (38) and SIRPα (39) were found to be expressed on mast cells and to inhibit FcεRI-dependent mast cell activation (39–41). The gp49B1 possesses two ITIMs, PIR-B and SIRPα four ITIMs and, by contrast with FcγRIIB which recruits the single-SH2 domain-containing phosphatases SHIP1 and SHIP2 (14), these three inhibitory receptors were shown to recruit the two-SH2 domain-containing phosphatases SHP-1 and/or SHP-2 (39, 42, 43). Having different inhibitory mechanisms (44), bispecific molecules that would coaggregate FcεRI with FcγRII and bispecific molecules that would coaggregate FcεRI with gp49B1, PIR-B or SIRPα could have additive, if not synergistic, inhibitory effects on IgE-induced mast cell activation, when used in combination.

## Acknowledgments

We are grateful for the advice and reagents from Dr Martin Glennie of Southampton, United Kingdom and Dr David Segal of National Cancer Institute, USA, that made this scientific work possible. We would like to thank Alison Tutt and Eric Mirabel for critically reviewing this manuscript.

## References

1. Turner H, Kinet JP. Signalling through the high-affinity IgE receptor FcεRI. Nature 1999;**402**:B24.
2. Reth MG. Antigen receptor tail clue. Nature 1989;**338**:383–384.
3. Jouvin MH, Adamczewski M, Numerof R, Letourneur O, Vallé A, Kinet JP. Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. J Biol Chem 1994;**269**:5918–5925.
4. Cambier JC. Antigen and Fc receptor signaling: the awsome power of the immunoreceptor tyrosine based activation motif (ITAM). J Immunol 1995;**155**:3281–3285.
5. Tamir I, Cambier JC. Antigen receptor signaling: integration of protein tyrosine kinase functions. Oncogene 1998;**17**:1353–1364.
6. Nadler MJ, Matthews SA, Turner H, Kinet J-P. Signal transduction by the high-affinity immunoglobulin E receptor Fc epsilon RI: coupling form to function. Adv Immunol 2000;**76**:325–355.
7. Daëron M. Fc receptor biology. Annu Rev Immunol 1997;**15**:203–234.
8. Daëron M, Latour S, Malbec O, Espinosa E, Pina P, Pasmans S et al. The same tyrosine-based inhibition motif, in the intracytoplasmic domain of FcγRIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. Immunity 1995;**3**:635–646.
9. Vivier E, Daëron M. Immunoreceptor tyrosine-based inhibition motifs. Immunol Today 1997;**18**:286–291.
10. Daëron M, Malbec O, Latour S, Arock M, Fridman WH. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. J Clin Invest 1995;**95**:577–585.
11. Malbec O, Fong D, Turner M, Tybulewicz VLJ, Cambier JC, Fridman WH et al. FcεRI-associated lyn-dependent phosphorylation of FcγRIIB during negative regulation of mast cell activation. J Immunol 1998;**160**:1647–1658.

12. Fong DC, Malbec O, Arock M, Cambier JC, Fridman WH, Daëron M. Selective in vivo recruitment of the phosphatidylinositol phosphatase SHIP by phosphorylated FcγRIIB during negative regulation of IgE-dependent mouse mast cell activation. Immunol Letters 1996;**54**:83–91.
13. Ono M, Bolland S, Tempst P, Ravetch JV. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor FcγRIIB. Nature 1996;**383**:263–266.
14. Bruhns P, Vély F, Malbec O, Fridman WH, Vivier E, Daëron M. Molecular basis of the recruitment of the SH2 domain-containing inositol 5-phosphatases SHIP1 and SHIP2 by FcγRIIB. J Biol Chem 2000;**275**:37 357–37 364.
15. Lesourne R, Bruhns P, Fridman WH, Daëron M. Insufficient phosphorylation prevents FcγRIIB from recruiting the SH2 domain-containing protein tyrosine phosphatase SHP-1. J Biol Chem 2001;**276**:6327–6336.
16. Daëron M, Vivier E. Biology of immunoreceptor tyrosine-based inhibition motif-bearing molecules. Cur Top Microbiol Immunol 1999;**244**:1–12.
17. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol 1999;**17**:875–904.
18. Choquet D, Partiseti M, Amigorena S, Bonnerot C, Fridman WH, Korn H. Cross-linking of IgG receptors inhibits membrane immunoglobulin-stimulated calcium influx in B lymphocytes. J Cell Biol 1993;**121**:355–363.
19. Scharenberg AM, El-Hillal O, Fruman DA, Beitz LO, Li Z, Lin S et al. Phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P3)/Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. EMBO J 1998;**17**:1961–1972.
20. Tamir I, Stolpa JC, Helgason CD, Nakamura K, Bruhns P, Daëron M et al. The RasGAP-binding protein p62dok is a mediator of inhibitory FcγRIIB signals in B cells. Immunity 2000;**12**:347–358.
21. Ujike A, Ishikawa Y, Ono M, Yuasa T, Yoshino T, Fukumoto M et al. Modulation of immunoglobulin (Ig)E-mediated systemic anaphylaxis by low-affinity Fc receptors for IgG. J Exp Med 1999;**189**:1573–1579.
22. Qiu WQ, de Bruin D, Brownstein BH, Pearse R, Ravetch JV. Organization of the human and mouse low-affinity FcγR genes: duplication and recombination. Science 1990;**248**:732–735.
23. Saito H, Ebisawa M, Tachimoto H, Shichijo M, Fukagawa K, Matsumoto K et al. Selective growth of human mast cells induced by Steel factor, IL-6, and prostaglandin E2 from cord blood mononuclear cells. J Immunol 1996;**157**:343–350.
24. Cho SH, Tam SW, Demissie-Sanders S, Filler SA, Oh CK. Production of plasminogen activator inhibitor-1 by human mast cells and its possible role in asthma. J Immunol 2000;**165**:3154–3161.
25. Valent P, Spanblochl E, Sperr WR, Zsebo KM, Agis H, Strobl H et al. Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. Blood 1992;**80**:2237–2245.
26. Greenman J, Tutt AL, George AJ, Pulford KAF, Stevenson GT, Glennie MJ. Characterization of a new monoclonal anti-FcγRII antibody, AT10, and its incorporation into a bispecific F(ab')2 derivative for recruitment of cytotoxic effectors. Mol Immunol 1991;**28**:1243–1254.
27. Sun LK, Liou RS, Sun NC, Gossett LA, Sun C, Davis FM et al. Transfectomas expressing both secreted and membrane-bound forms of chimeric IgE with anti-viral specificity. J Immunol 1991;**146**:199–205.
28. Lamoyi E, Nisonoff A. Preparation of F(ab')2 fragments from mouse IgG of various subclasses. J Immunol Methods 1983;**56**:235–243.
29. Glennie MJ, McBride HM, Worth AT, Stevenson GT. Preparation and performance of bispecific F(ab' gamma)2 antibody containing thioether-linked Fab' gamma fragments. J Immunol 1987;**139**:2367–2375.
30. Van Toorenenbergen AW, Aalberse RC. IgG4 and passive sensitization of basophil leukocytes. Int Arch Allergy Appl Immunol 1981;**65**:432–440.
31. Zhu D, Kepley C, Zhang LM, Zhang K, Saxon A. A novel human immunoglobulin Fcγ-Fcε bifunctional fusion protein inhibits FcεRI-mediated degranulation. Nature Medicine 2002;**8**:518–521.
32. Kulczycki A, Metzger H. The interaction of IgE with rat basophilic leukemia cells. II: quantitative aspects of the binding reaction. J Exp Med 1974;**140**:1676–1695.
33. Stuart SG, Simister NE, Clarkson SB, Kacinski BM, Shapiro M, Mellman I. Human IgG Fc receptor (hFcRII; CD32) exists as multiple isoforms in macrophages, lymphocytes and IgG-transporting placental epithelium. EMBO J 1989;**8**:3657–3666.
34. Maurer D, Fiegiger E, Reininger B, Wolffwiniski B, Jouvin M-H, Kilgus O et al. Expression of a functional high affinity immunoglobulin E receptor (FcεRI) on monocytes of atopic individuals. J Exp Med 1994;**179**:745–750.
35. Gounni AS, Lamkhioued B, Ochial K, Tanaka Y, Delaporte E, Capron A et al. High-affinity IgE receptor on eosinophils is involved in defence against parasites. Nature 1994;**367**:183–186.
36. Capron M, Morita M, Woerly G, Lengrand F, Gounni AS, Delaporte E et al. Differentiation of eosinophils from cord blood cell precursors: kinetics of Fc epsilon RI and Fc epsilon RII expression. Int Arch Allergy Immunol 1997;**113**:48–50.
37. Arm JP, Nwankwo C, Austen KF. Molecular identification of a novel family of human Ig superfamily members that possess immunoreceptor tyrosine-based inhibition motifs and homology to the mouse gp49B1 inhibitory receptor. J Immunol 1997;**159**:2342–2349.
38. Kubagawa H, Burrows PD, Cooper MD. A novel pair of immunoglobulin-like receptors expressed by B cells and myeloid cells. Proc Natl Acad Sci USA 1997;**94**:5261–5266.
39. Liénard H, Bruhns P, Malbec O, Fridman WH, Daëron M. Signal regulatory proteins negatively regulate immunoreceptor-dependent cell activation. J Biol Chem 1999;**274**:32 493–32 499.
40. Katz HR, Vivier E, Castells MC, McCormick MJ, Chambers JM, Austen KF. Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation upon coligation with FcεRI. Proc Natl Acad Sci USA 1996;**93**:10 809–10 814.
41. Uehara T, Bléry M, Kang D-W, Chen C-C, Ho LH, Gartland GL et al. Inhibition of IgE-mediated mast cell activation by the paired Ig-like receptor PIR-B. J Clin Invest 2001;**108**:1041–1050.

42. Lu-Kuo JM, Joyal DM, Austen KF, Katz HR. gp49B1 inhibits IgE-initiated mast cell activation through both immunoreceptor tyrosine-based inhibitory motifs, recruitment of src homology 2 domain-containing phosphatase-1, and suppression of early and late calcium mobilization. J Biol Chem 1999;274:5791–5796.

43. Blériy M, Kubagawa H, Chen CC, Vely F, Cooper MD, Vivier E. The paired Ig-like receptor PIR-B is an inhibitory receptor that recruits the protein-tyrosine phosphatase SHP-1. Proc Natl Acad Sci USA 1998;95:2446–2451.

44. Gupta N, Scharenberg AM, Burshtyn DN, Wagtmann N, Lioubin MN, Rohrschneider LR et al. Negative signaling pathways of the killer cell inhibitory receptor and Fc gamma RIIb1 require distinct phosphatases. J Exp Med 1997;186:473–478.